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13. ABSTRACT *(Maximum 200*

Tumor angiogenesis is the process by which a growing tumor mass recruits the new blood vessels required for its continued growth, and through which the tumor can spread to distant sites. This proposal focuses on the development of inhibitors of a particular angiogenic factor, thymidine phosphorylase, by targeting the enzymatic activity of TP, since it has been found to be required for angiogenesis. In the past year, several compounds were synthesized as potential TP inhibitors, including one having high potency as an enzyme inhibitor (IC_{50} = 180 nM). TP was found to induce human endothelial cell migration *in vitro*, a process found to require both a TP gradient and the presence of thymidine. The TP-mediated endothelial cell migration was blocked by TP inhibitors, and this was found to be specific in that the actions of a different angiogenic factor, VEGF (vascular endothelial cell growth factor), were not blocked.

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Ed Schmitz 8/31/99

PI - Signature Date

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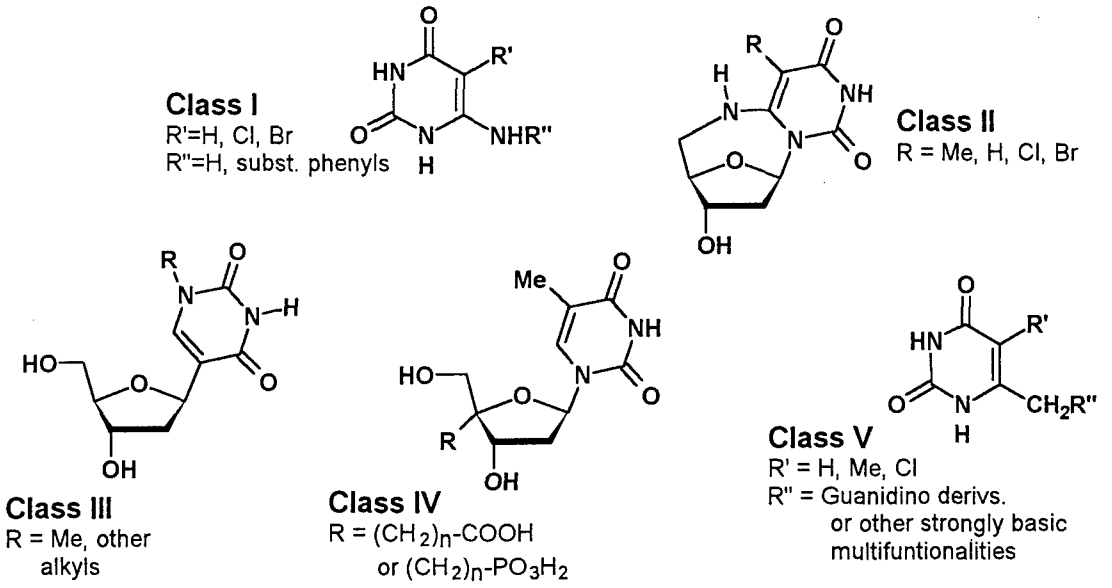
Introduction. There is a need for alternative approaches to treat metastatic breast cancer. One rapidly developing area of investigation is the study of tumor angiogenesis, the process by which a growing tumor mass recruits the new blood vessels required for its continued growth, and through which the tumor can spread to distant sites. [1-2] Most studies have demonstrated the importance of angiogenesis in the progression of human breast malignancies and found the extent of vascularization to be correlated with tumor size, frequency of metastasis, and poor prognosis.[3-8] Several polypeptides and growth factors that are produced by breast cancer epithelial and stromal cells have been identified as having endothelial cell mitogenicity and angiogenic activity.[1,9] Our studies are focusing on the angiogenic factor PD-ECGF, based on recent evidence demonstrating its role in experimental and human cancer, and the finding that PD-ECGF is identical to human thymidine phosphorylase (TP).[10-12] When transfected into NIH 3T3 cells, TP was found to increase the vascularization of tumors growing in nude mice after sc inoculation.[13] Similarly, overexpressing TP in MCF7 breast carcinoma cells markedly increased tumor growth *in vivo*, although it had no effect on the growth of the cells *in vitro*.[14] Western blot analysis of primary human breast tissue showed that TP expression was elevated in the tumors compared to the normal tissue[14], a finding which provides clinical relevance to the transfection experiments. Studies suggest that the angiogenic and endothelial cell chemotactic activities of PD-ECGF are dependent upon its enzymatic activity, and this has been confirmed with site-directed mutagenesis studies.[14,15-17] Of the angiogenic factors identified to date, TP is the only one in which an enzymatic activity of the factor is required for angiogenic activity. These observations serve as the basis for our hypothesis that inhibition of the catalytic activity of TP will also block its angiogenic properties. The purpose of this study is to design and synthesize highly potent inhibitors of TP enzymatic activity, and determine their antiangiogenic and antitumor activities in *in vitro* and *in vivo* model systems.

Technical objective 1: Synthesis of inhibitors of TP.

Our original proposal suggested the synthesis of four structurally distinct classes of pyrimidines or pyrimidine nucleosides for evaluation as potential TP inhibitors (**I-IV** shown in Fig. 1). In view of some significant new developments in the area of inhibitors as antiangiogenic agents we had also incorporated in last years report a new series of analogs (**class V**) which were structurally similar to **class I** but required separate synthetic methodologies.

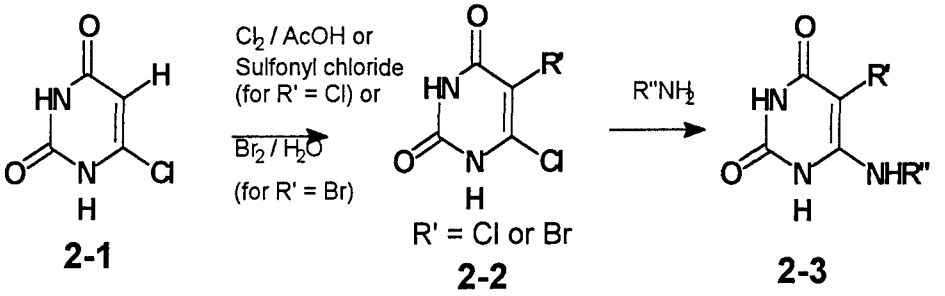
FIGURE 1.



We describe below progress during the previous year towards completion of the five targeted classes of compounds.

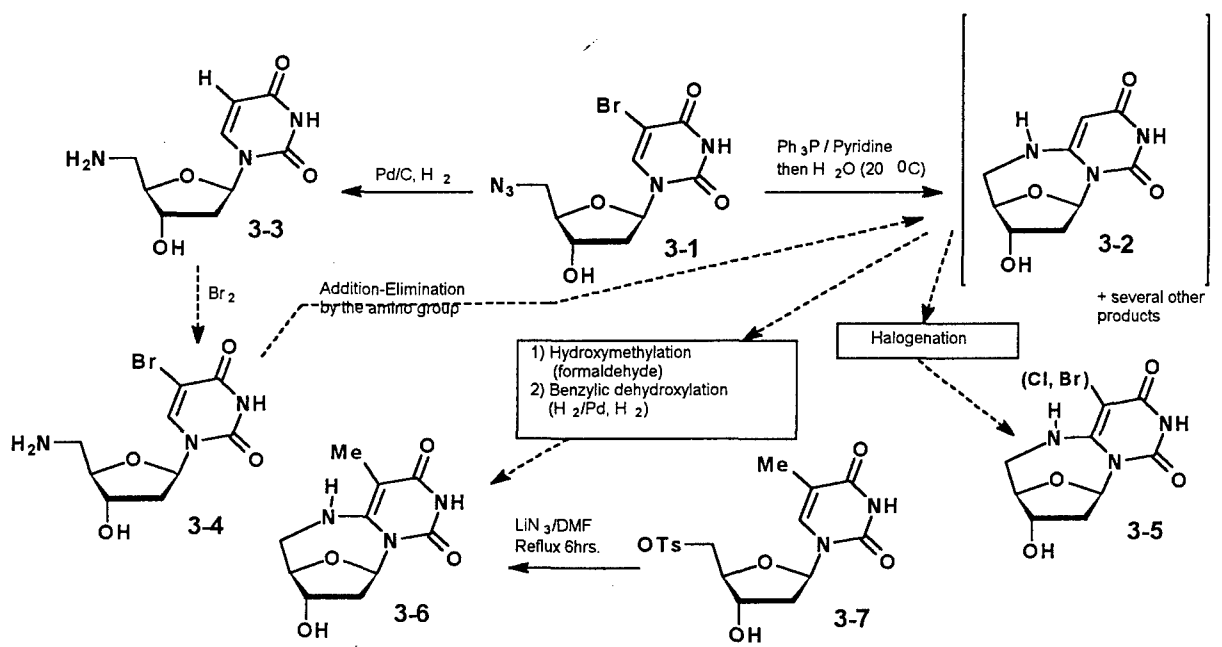
We had already reported earlier on the synthesis of several new derivatives of **class I** (see Fig.1, R′ = H, R" = substituted phenyl derivatives) which exhibited moderate TP inhibitory activities, with IC_{50} values in the range of 30 - 275 μM. Halogenation of the 5-position of these compounds was attempted in order to obtain those targets where R′ = Cl or Br since, as was described in the original application, it is anticipated that such derivatives would exhibit enhanced activities because of their closer stereoelectronic resemblance to thymine. In most instances, chlorination or bromination at C-5 was also accompanied by partial halogenation of the phenyl substituent at C-6 thus affording complex mixtures of products. It was decided therefore to prepare the desired 5-halogenated derivatives by reaction of already 5-halogenated 6-chlorouracil intermediates (such as **2-2**, in fig 2) with the appropriate amines as we originally suggested as a back-up approach. As illustrated in figure 2, work is now in progress for the halogenation of 6-chlorouracil **2-1** (readily accomplished via known procedures) and for the displacement of the 6-chloro function by various anilino and other amines (as already carried out for derivatives with R′ = H).

FIGURE 2.



Access to the targeted 5',6-cyclouridine derivatives of **class II** by selective reduction of the azido function of intermediate **3-1** (Fig. 3) to give **3-4** has also been attempted in the expectation that this product could be made to undergo addition-elimination to the final desired product **3-2**. Catalytic hydrogenation over Pd/C however was found to give **3-3** undoubtedly produced by the concurrent hydrogenolytic debromination at C-5. The desired product **3-2** (soon to be tested for biological activity) could be obtained, albeit in poor yields, by reduction of **3-1** with triphenyl phosphine. Its formation, however, was accompanied by several undesirable by-products. A more promising strategy therefore lies in the bromination of **3-3** (more readily obtained by the direct 5'-azidation of 2'-deoxyuridine) to afford **3-4** and, after ring closure, **3-2**. The latter would then serve as the major intermediate to all remaining targets (**3-5** or **3-6**) as outlined in fig. 3. An attractive alternate route to the desired 5-Me derivative **3-6** (reported in the literature by A. Matsuda and associates) may also be employed. It is based on displacement of the 5'-O-tosyl function of thymidine derivative **3-7** by azide followed by an *in situ*, thermally induced 1,3-dipolar cycloaddition of the azido

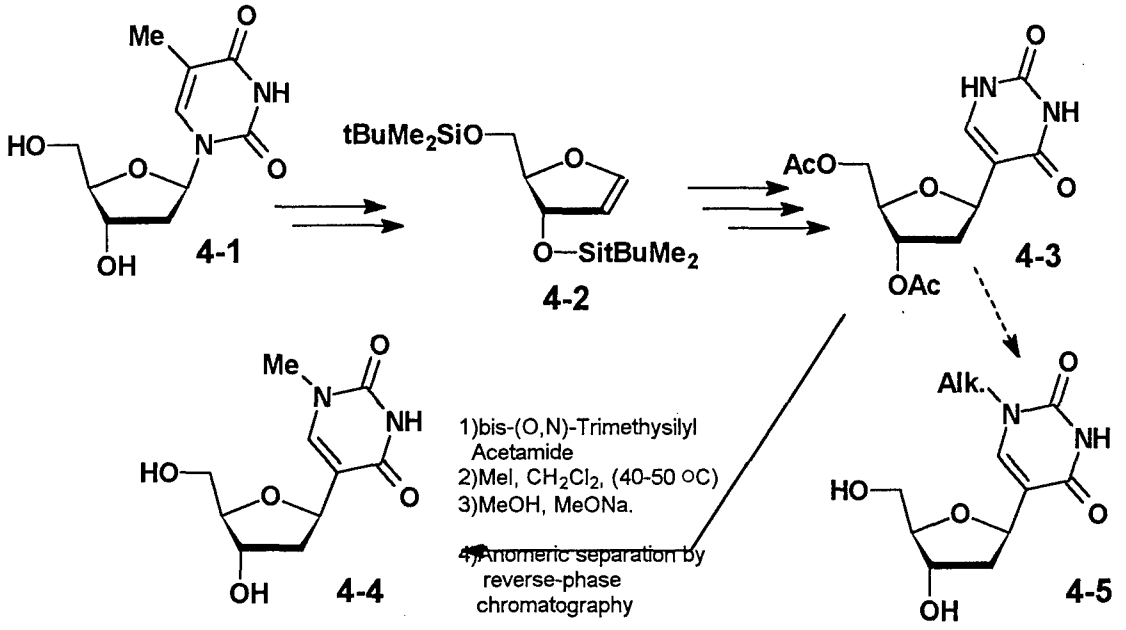
FIGURE 3.



function onto the 5,6 pyrimidine double bond and spontaneous elimination of N_2. Work is now in progress to achieve these goals.

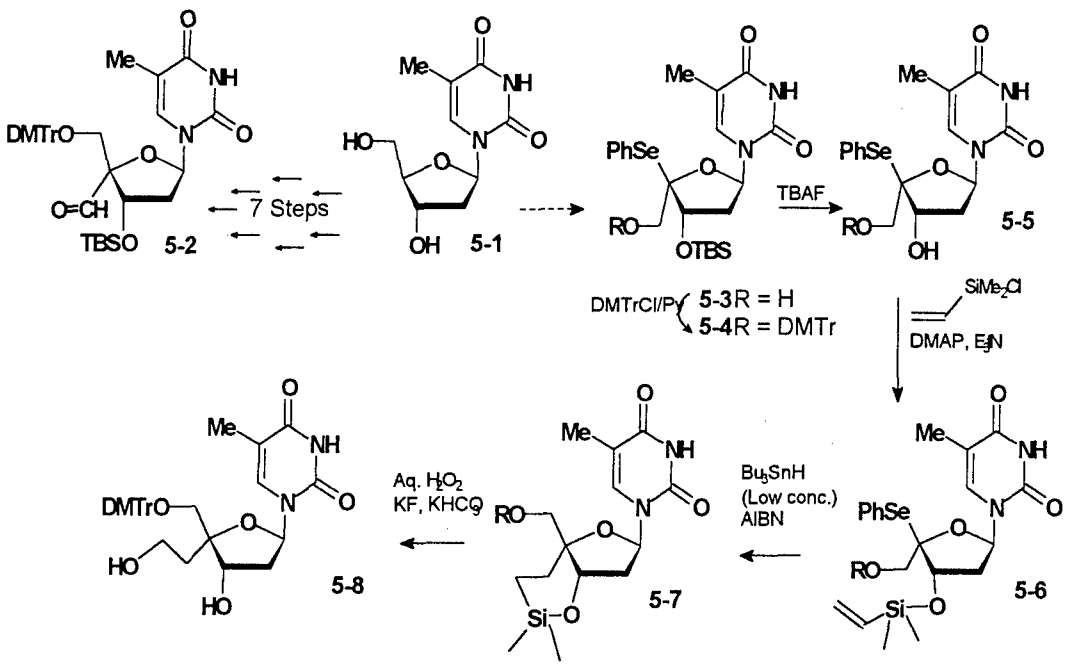
As we had reported earlier, the thymidine analog **4-4** which is the major representative of **class III** had been synthesized as outlined in fig. 4 and was found to be inactive. Efforts have been expended in the past year to resynthesize key intermediate **4-3** so as to be able to make new derivatives with groups other than the N-methyl to be assayed for enhanced activity. This work is now in progress.

FIGURE 4.



We had reported earlier on the synthesis of compound **5-2** (fig. 5), a key intermediate in the projected synthesis of the members of **class IV**. Further efforts to utilize it for subsequent functionalization of the side chain at C-4′ have been thwarted by its relative instability and the length of the multistep procedure required to obtain it. We have therefore begun to exploit an alternative approach based on the recently reported regio- and stereospecific method for introducing a 2-hydroxyethyl group as a C-4′ carbon-branched function in thymidine (**5-1**). The method (outlined in fig. 5) utilizes the 4′-(phenylseleno)thymidine derivative **5-3** (prepared in several high yielding steps from thymidine) for introducing a dimethylvinylsilyl group as a radical acceptor tether (as in **5-6**) followed by a free radical reaction (Bu_3SnH/AIBN) and a Tamao oxidation to give the desired product **5-8**. The latter is ideally suited as synthetic substitute for intermediate **5-2** since its primary hydroxyl function can undergo selective functionalization either directly or indirectly to an aldehyde followed by further extension via a Wittig reaction (or a Horner modification) to afford the desired phosphonates or carboxylates proposed as targets of **class IV**.

FIGURE 5.

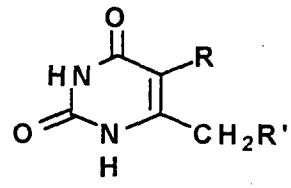


The largest portion of our efforts have been spent on the synthesis of new members of class V, because the most active inhibitors of TP known to date belong to this class of uracil analogues. Table 1 tabulates a new set of compounds prepared recently. All were prepared in 60-90% yields by reacting the corresponding 5-substituted (R=H, Me, Cl) 6-chloromethyluracil with a 1.5-2 fold molar excess of the amine in refluxing methanol for 3-4 hrs. Addition of HCl gas to the precipitated product and filtration afforded the hydrochloride form while neutralization with aqueous sodium bicarbonate afforded the free base form. A recent report confirmed the activity of a different (but structurally related) TP inhibitor which also had antitumor activity *in vivo*[18].

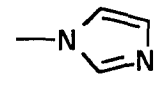
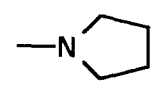
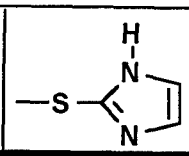
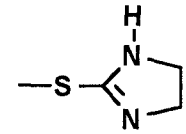
Technical objective 2: Evaluation of target compounds as TP inhibitors and for effects on endothelial cells.

TP activity was measured in assays which contained 0.2 M KH_2PO_4 (pH 7.8), 0.2 mM [5′-^{3}H] thymidine (1 μCi), human TP (5 ng), and several different concentrations of the inhibitor being tested. Reactions were stopped after 30 min at 37° by the addition of ice-cold TCA containing activated charcoal. After centrifugation, an aliquot of the supernatant was counted in a liquid scintillation counter. IC_{50} values for each compound shown in Table 1 were calculated from data from at least 3 determinations.

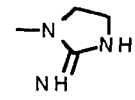
Table 1.

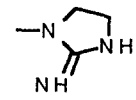


Structure Activity Relationship of some Cpds of **Class V** Uracil Derivatives as TP Inhibitors prepared in our laboratories (F.W. / form and activity against the human enzyme)

R \ R'				
H	192.17 (Free base) IC $_{50}$ ~ **300** μM	195.22 (Free base) IC $_{50}$ ~ **300** μM	260.69 (HCl salt) IC $_{50}$ ~ **200** μM	262.71 (HCl salt) IC $_{50}$ ~ **300** μM
CH_3	206.20 (Free base) IC $_{50}$ ~ **300** μM	209.24 (Free base) IC $_{50}$ ~ **5** μM	274.72 (HCl salt) IC $_{50}$ ~ **400** μM	276.74 (HCl salt) IC $_{50}$ ~ **200** μM
Cl	263.08 **85-32** (HCl salt) IC $_{50}$ ~ **190nM**	229.66 ——	295.14 (HCl salt) IC $_{50}$ ~ **300** μM	297.15 ——

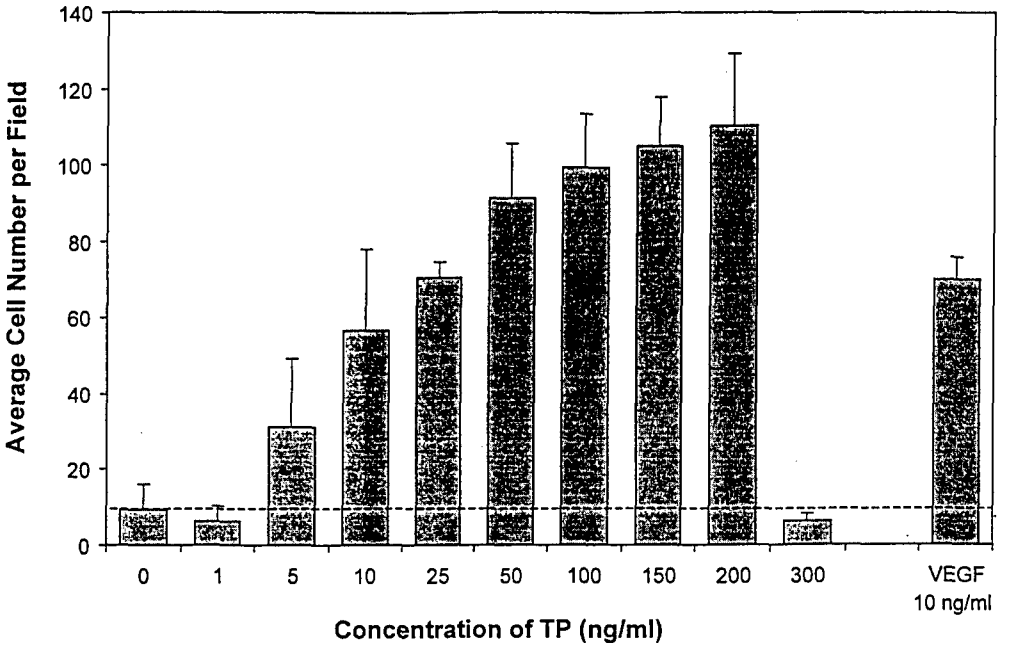
Recently reported activities of structurally similar analogs

R = Cl, R' = 
Ki =13 nM

R = Br, R' = 
Ki = 30 nM

We have also begun our studies with human umbilicial vein endothelial cells (HUVEC) in an endothelial cell migration assay. Initial experiments to optimize and characterize the culture system and response to TP are shown below.

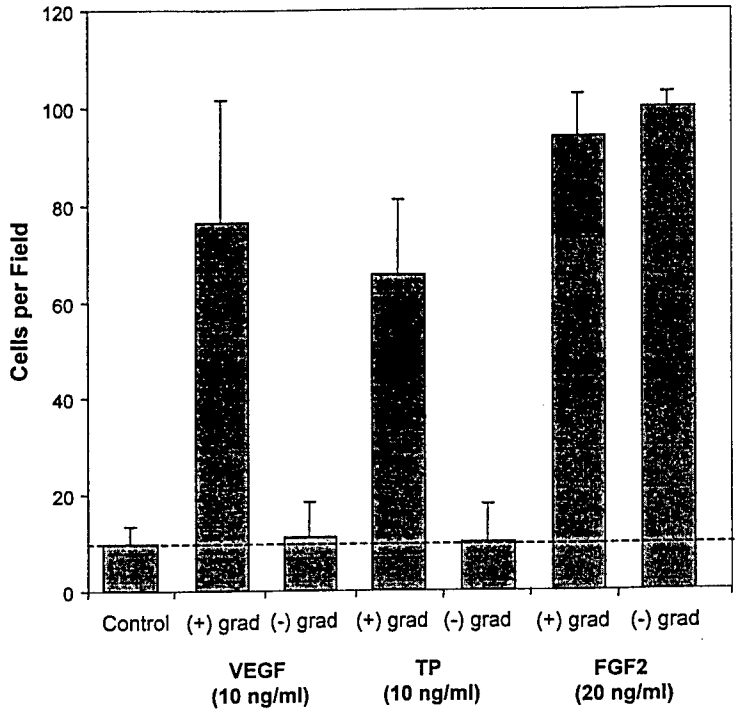
FIGURE 6 (next page). Dose dependent induction of HUVEC migration by TP. Human umbilical vein endothelial cells (isolated immediately prior to use) were cultured in the top well of 24 well plates which had an 8 micron pore size polycarbonate, collagen-coated, filter cell culture insert. TP or VEGF (vascular endothelial cell growth factor; used as a positive control) were added to the bottom chamber were added at the indicated concentrations. After 4 hours at 37°, cells on the bottom of the filters (i.e. endothelial cells which had migrated through the pores) were fixed, stained, and counted manually under a microscope. Data for all figures are means ± SD.



Conclusion: TP induces endothelial cell migration in a concentration-dependent manner.

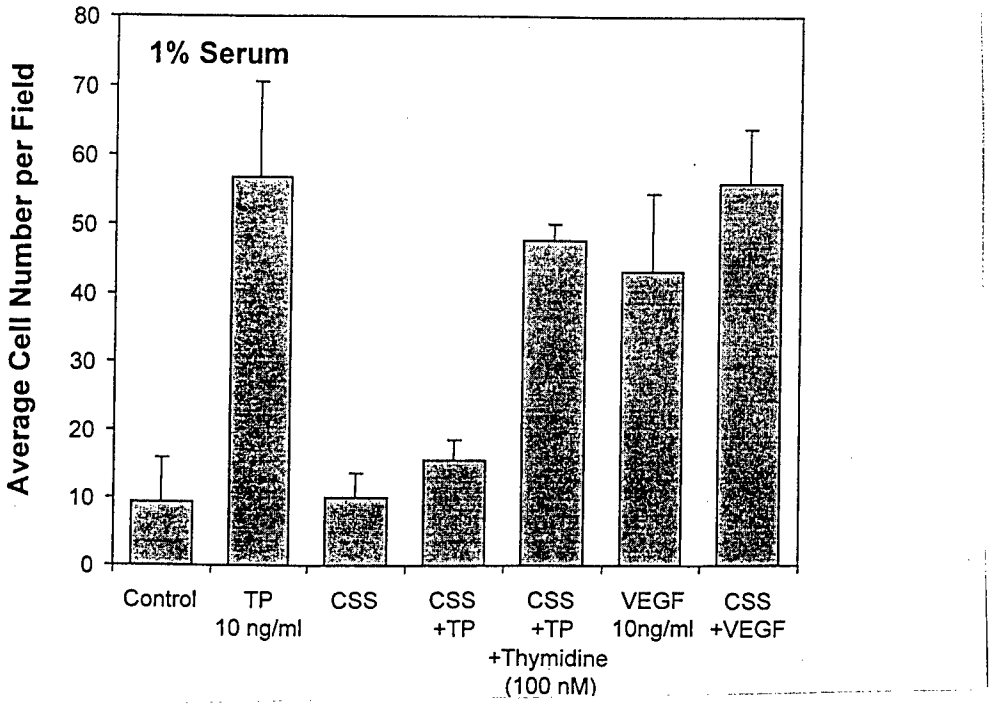
In the next two series of experiments, we further characterized the mechanism for the stimulation of endothelial cell migration by TP. In the experiments shown in Figure 7, the TP was added at the same concentration as in Figure 6, but it was added to both sides of the chamber, thereby retaining TP enzyme activity but eliminating the gradient for TP. VEGF and FGF2 (bFGF or basic fibroblast growth factor) were included as controls in these experiments. As shown in Fig. 7, no migration of endothelial cells was seen when the gradients for TP or VEGF were eliminated, confirming that the nature of the migration induced can be described as chemotactic. In contrast, FGF was active both in the presence and absence of a gradient, indicating its action is to induce increased chemokinetic rather than chemotactic activity.

Figure 7 (next page). TP is a chemotactic mediator of endothelial cell migration. Experiments were carried out with HUVEC cells as described in the legend to figure 6, except in instances in which TP was added to both the top and bottom wells of the chambers (labeled "(-) grad"). VEGF and FGF2 were added to separate wells as controls.



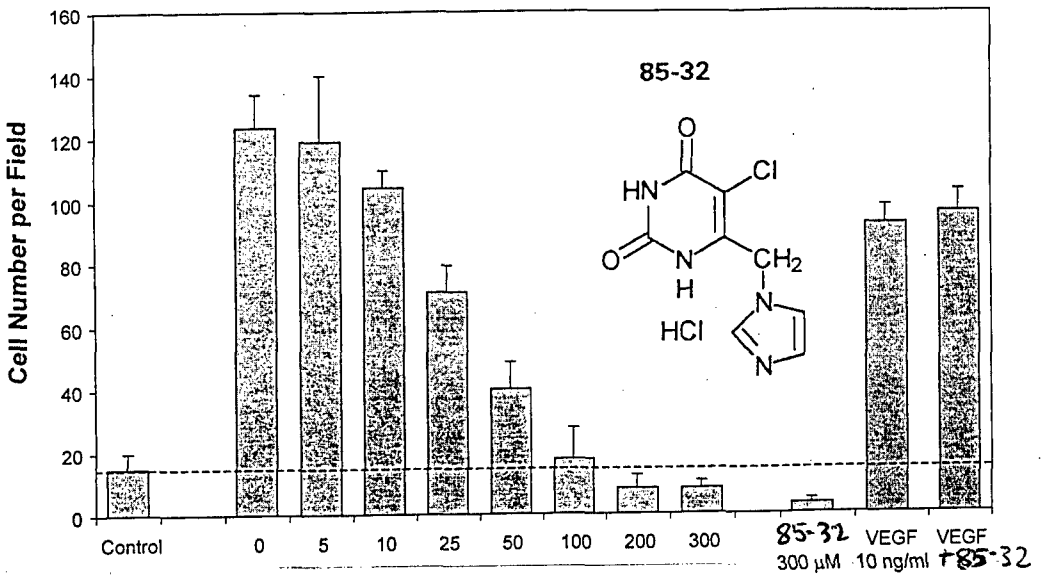
We also evaluated the role of thymidine in the actions of TP. All experiments shown in Figs. 6 & 7 were done in medium containing 1% serum, which contains thymidine at a concentration between 5 and 10 μM. When the serum was first treated with charcoal to remove the thymidine (CSS or charcoal-stripped serum in Fig. 8), the actions of TP were eliminated. Full migration-inducing activity could be restored to the CSS, however, by the addition of thymidine to a final concentration of 100 nM, equivalent to that found in medium containing 1% serum. We conclude from this experiment that thymidine must be present for TP to exert its actions. In contrast, VEGF induction of migration was not attenuated when CSS was used, indicating that thymidine or other molecules which bound to charcoal were not required for its actions.

FIGURE 8. TP requires thymidine to stimulate endothelial cell migration. Experiments were carried out with HUVEC cells as described in the legend to figure 6, except in instances in which charcoal-stropped serum (CSS) was used in place of 1% fetal bovine serum. TP was added to bottom wells of the chambers at a concentration of 10 ng/ml, and thymidine was used when indicated at a concentration of 100 nM.



Having confirmed the ability of TP to induce endothelial cell migration, we next examined the effect of one of the inhibitors which we had synthesized (5-chloro-6(1-imidazolylmethyl) uracil hydrochloride; structure 85-32 in Table 1, above). We found that 85-32 inhibited in a concentration-dependent manner the TP-mediated induction of endothelial cell migration. The inhibition was specific for TP, as 85-32 had no effect on VEGF-mediated chemotaxia.

Figure 9. Dose-dependent inhibition of TP-mediated endothelial cell migration by 85-32. These assays were done as above (legend to Fig. 6) with 100 ng/ml TP or 10 ng/ml VEGF and increasing concentrations of 85-32 (5 - 300 μM), as indicated. Control wells were without TP or VEGF.



Technical objective 3: *In vivo* assays. We have begun experiments toward this objective. MCF7 human breast carcinoma cells were transfected with a cDNA for either human TP (clone pCMV-TPneo) or a control vector (pCMV-neo1) with the use of lipofectamine. Cells were cloned in the presence of G418 (geneticin), and after several weeks, individual clones were selected and expanded in liquid culture in the continuous presence of G418. These stably transfected cell lines were evaluated for TP mRNA expression, and one high-expressing line was selected for further study. In the figure to the right are shown the result of a RT- PCR analysis of RNA from control MCF7 cells (left lane), MCF7 cells transfected with the control vector (middle lane), or MCF7 cells transfected with the TP-containing vector (right lane). The lower bands are the fragments corresponding to the TP cDNA, and the upper bands are a GAPDH-fragment, used as a control.

We have recently begun an experiment in which the stably-transfected MCF7 cells (10^6 cells) containing either the control or TP vector were injected into the mammary fat pad of groups of Balb/c nude mice. We are now monitoring these mice for tumor growth.

Conclusions. A number of inhibitors of TP have been synthesized, including one (85-32) which is relatively potent (K_i < 200 nM). This compound can serve as a starting point to synthesize analogs to try to further increase TP-inhibitory potency. An *in vitro* assay of human endothelial cell migration in response to TP has been well characterized, and 85-32 has been shown to be active in this assay. This assay will continue to be utilized to identify new TP inhibitors and to further define their mechanisms of action. *In vivo* studies have begun and will eventually be used to evaluate the TP inhibitors under development.

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DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

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PHYLLIS M. RINEHART
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